Drosophila grapes/CHK1 mutants are defective in cyclin proteolysis and coordination of mitotic events

Tin Tin Su*†, Shelagh D. Campbell‡ and Patrick H. O'Farrell*

The *Drosophila grapes* (*grp*) gene, which encodes a homolog of the *Schizosaccharomyces pombe* Chk1 kinase, provides a cell-cycle checkpoint that delays mitosis in response to inhibition of DNA replication [1]. Grp is also required in the undisturbed early embryonic cycles: in its absence, mitotic abnormalities appear in cycle 12 and chromosomes fail to fully separate in subsequent cycles [2,3]. In other systems, Chk1 kinase phosphorylates and suppresses the activity of Cdc25 phosphatase: the resulting failure to remove inhibitory phosphate from cyclin-dependent kinase 1 (Cdk1) prevents entry into mitosis [4,5]. Because in *Drosophila* embryos Cdk1 lacks inhibitory phosphate during cycles 11–13 [6], it is not clear that known actions of Grp/Chk1 suffice in these cycles. We found that the loss of *grp* compromised cyclin A proteolysis and delayed mitotic disjunction of sister chromosomes. These defects occurred before previously reported *grp* phenotypes. We conclude that Grp activates cyclin A degradation, and functions to time the disjunction of chromosomes in the early embryo. As cyclin A destruction is required for sister chromosome separation [7], a failure in Grp-promoted cyclin destruction can also explain the mitotic phenotype. The mitotic failure described previously for cycle 12 *grp* embryos might be a more severe form of the phenotypes that we describe in earlier embryos and we suggest that the underlying defect is reduced degradation of cyclin A.

Addresses: *Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, California 94143-0448, USA. †MCD Biology, University of Colorado, Boulder, Colorado 80309-0347, USA.

‡Present address: Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9.

Correspondence: Patrick H. O'Farrell
E-mail: ofarrell@cgl.ucsf.edu

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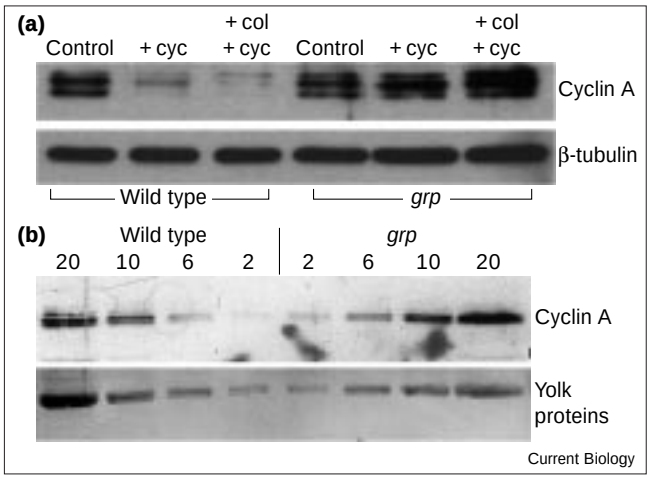
Results and discussion

When syncytial embryos are exposed to a protein synthesis inhibitor, cycloheximide, nuclear cycles arrest in interphase and cyclin A levels decline ([6]; Figure 1, wild type, + cyc) whereas cyclin B is stable under these conditions [6]. This suggests that a steady state of synthesis and degradation maintains the interphase levels of cyclin A during the syncytial divisions. We found that *grp* mutants are deficient in cyclin A turnover in the presence of cycloheximide (Figure 1, *grp*, + cyc). This defect was seen in embryos in cycles 4–8, earlier than other reported *grp* phenotypes and is therefore unlikely to be secondary to these phenotypes. We infer that Grp normally destabilizes cyclin A.

In wild-type embryos, cyclin A is unstable not only in interphase but also during mitotic arrest caused by microtubule destabilization. Thus, in embryos treated with colchicine, blocking protein synthesis with cycloheximide leads to a decline in cyclin A levels ([6]; Figure 1,

Figure 1



Cyclin A turnover and abundance in *grp*^{1} (*grp*) and wild-type embryos. (**a**) Defective cyclin A turnover in *grp* embryos. Syncytial-stage embryos (30–70 min old, cycles 4–8) were either untreated (control), or treated with cycloheximide (+ cyc), or colchicine and cycloheximide (+ col, + cyc), homogenized and extracts separated on denaturing gels and western blotted for expression of cyclin A and β-tubulin. The latter provided a loading control. (**b**) Higher levels of cyclin A in *grp* embryos. Extracts from wild-type and *grp* embryos (volumes loaded in each lane indicated in μl) were separated on denaturing gels and western blotted for cyclin A expression. Ponceau staining of yolk proteins indicated equal loading in corresponding lanes between wild-type and *grp* samples. In each case, the cyclin A signal was higher in the *grp* sample than in an equal volume of the wild-type sample. We estimate that *grp* embryos have about 1.5–3-fold higher cyclin A levels than wild-type embryos. For example, the signal in the 2 μl lane for the *grp* sample is higher than in the corresponding lane for the wild type, but comparable to the signal in the 6 μl lane for the wild type. The signal in the 6 μl lane for *grp* is higher than in the corresponding lane for the wild type, but is comparable to the signal in the 10 μl lane for the wild type.

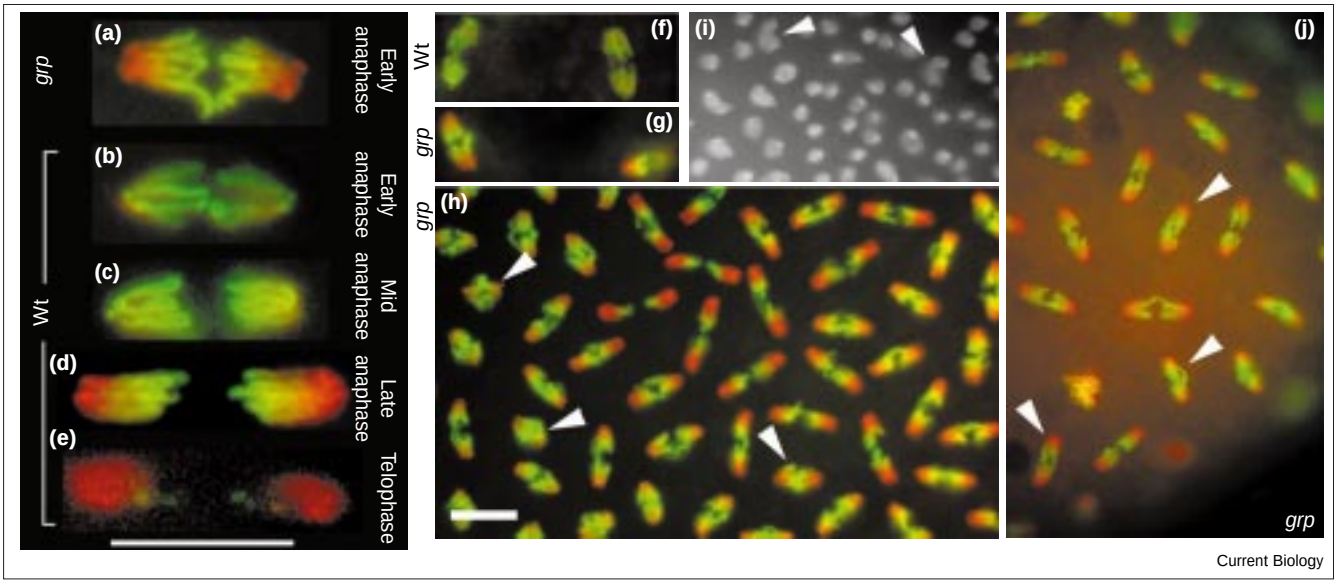
wild type, + col, + cyc), whereas cyclin B is stable under these conditions [6]. We found that *grp* embryos are compromised for cyclin A proteolysis at such a colchicine-induced mitotic arrest (Figure 1, *grp*, + col, + cyc). As for interphase destruction of cyclin A, this defect was seen during early syncytial cycles, before the onset of other reported *grp* phenotypes. We conclude that Grp promotes cyclin A degradation in colchicine-arrested early embryos.

If normal levels of cyclin A are maintained by a steady state of synthesis and destruction, we would expect the levels of cyclin A to be high in *grp* embryos as a result of increased stability. Western blotting showed that *grp* embryos had slightly higher levels of cyclin A than wild-type embryos (1.5–3-fold; Figure 1b). Although this relatively small increase suggests that significant degradation of cyclin A still occurs in *grp* embryos, this degradation is not apparent at the arrests induced by cycloheximide or by colchicine.

As Grp is required for destruction of cyclin A in an arrested mitosis, we determined whether mitosis was disrupted in *grp* embryos. The mitosis-specific phosphorylation of histone H3 (PH3) apparently acts as an *in vivo* reporter for cyclin/Cdk activity and its disappearance at the end of mitosis requires cyclin destruction [8]. During syncytial cycles of wild-type embryos, PH3 staining is continuous along the length of the chromosome arms from metaphase until late anaphase, when loss of the epitope near kinetochores leads to graded staining ([8]; Figure 2b–d). In embryos from grp^1 homozygous females or from grp^1/Df females, the gradient of PH3 was seen on chromosomes in early anaphase (Figure 2a,g,h). Thus, PH3 loss is advanced with respect to chromosome segregation in *grp* embryos. This defect was seen at the earliest cycles scored (cycle 4 in grp^1/grp^1 and cycle 3 in grp^1/Df), well before the onset of previously reported defects in *grp* embryos (cycle 11).

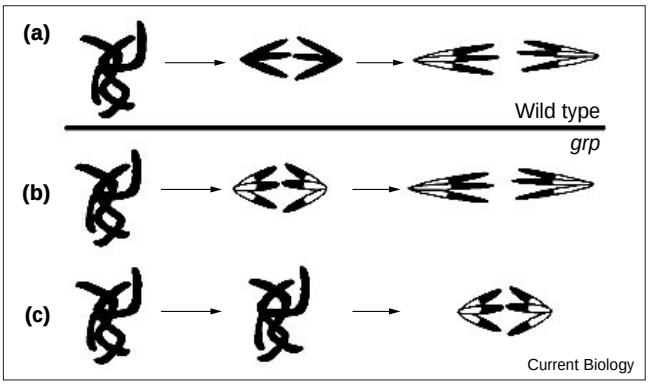
The loss of PH3 during early anaphase in *grp* embryos could be due to the premature loss of PH3 (Figure 3b), a scenario opposite to that expected for a mutation that stabilizes cyclin A during mitosis. Alternatively, timely loss of PH3 staining but delayed chromosome separation would produce the same mis-coordination (Figure 3c). Analysis of *grp* embryos supports the latter hypothesis: we found an

Figure 2



Histone H3 dephosphorylation begins at earlier stages of chromosome segregation in *grp* mutants. Syncytial-stage Sevelen (wild type, wt) or *grp* embryos were fixed and stained for DNA (red) and with an antibody to PH3 (green). (**a–e**) Timing of mitotic loss of PH3 staining in a *grp* embryo compared with the progression of PH3 loss in wild-type mitoses; (b–e) are reproduced from [8]. Loss of PH3 staining from the leading ends of chromosome arms in the wild type occurs as the chromosomes approach the spindle poles (d) whereas loss of PH3 in *grp* embryos is detected when chromosomes disjoin (a). Mitotic figures are from the M phase of cycle 10 (M10) and 11 (wild type) or M10 (*grp*). (**f**,**g**) PH3 loss in wild-type embryos compared with *grp* embryos during M9. (**h**) M12 in a *grp* embryo. The chromosomes of all mitotic figures show a gradient of PH3 though anaphase chromosome separation is less than that at which such PH3 gradients occur in a wild-type mitosis (see panel d). Severely mis-coordinated mitotic figures are indicated by arrowheads. (**i**) A portion of a later-stage (about cycle 12) *grp* embryo in interphase, stained for DNA: polyploid nuclei are discernible (arrowheads). These polyploid nuclei might arise if the mis-coordination of mitosis reaches a point at which the chromosomes decondense when anaphase separation of DNA masses is not yet adequate to define separate nuclei. (**j**) Mitotic figures (M10 or 11) in an embryo from a grp^1/Df mother. As seen for the embryos from grp^1/grp^1 mothers (all other *grp* panels), the chromosomes in (j) show early loss of PH3 (arrowheads indicate particularly severe examples). Df is deficiency chromosome Df(2L)H20 (Bloomington Stock Center) that carries breakpoints at 36A08-09;36E-01-02 and therefore lacks the *grp* gene. The bar represents 10 μm.

Figure 3



Chromosome segregation in (a) the wild type and (b,c) the two possible scenarios in *grp* embryos. (a) In wild-type mitoses, loss of PH3 (black) from chromosomes begins in late anaphase to reveal the DNA stain (white). (b,c) In *grp* embryos, either (b) PH3 loss is premature or (c) chromosome segregation is delayed but PH3 loss begins at about the normal time, resulting in histone H3 dephosphorylation at earlier stages of chromosome segregation.

increase in the ratio of embryos with unsegregated chromosomes (prophase/metaphase) to those with segregated chromosomes (anaphase/telophase; Table 1). We conclude that Grp is required for timely chromosome segregation in syncytial mitoses.

The mitotic phenotype in *grp* embryos can be understood as follows. Ordinarily, the mitotic cyclins are degraded in a sequence during exit from mitosis: cyclin A is degraded before the metaphase–anaphase transition, cyclin B is degraded at the beginning of anaphase and cyclin B3 towards the end of anaphase [7]. The disappearance of PH3 can be prevented by stabilization of any of these mitotic cyclins [8]; thus, it appears that loss of PH3 marks the completion of this sequence. We suggest that *grp* embryos are specifically defective in the early initiation of cyclin A degradation but they do degrade cyclin A, perhaps in conjunction with the B cyclins. Eventual destruction of cyclin A would explain the ability of *grp*-deficient nuclei to exit mitosis and lose PH3 staining, events that can be inhibited by stable cyclin A [7,8]. Destruction of cyclin A in conjunction with the B cyclins would explain why the length of mitosis is not increased in *grp* embryos [1,9]. Because the expression of a stable form of cyclin A prevents chromosome disjunction [7,8], we suggest that the failure to degrade cyclin A early during mitosis in *grp* embryos delays chromosome disjunction. The delay in chromosome separation abbreviates anaphase and, when the abbreviation is severe, decondensation of chromosomes and entry into the next interphase occurs before the separating chromosomes reach the spindle poles (Figure 2h–j).

Table 1

Mitotic indices in wild-type and *grp* embryos.

	Percentage of embryos in M phase (SD/*n*)	Percentage of embryos in M phase before chromosome segregation (SD)	Percentage of embryos in M phase after chromosome segregation (SD)	*n*
Wild type	58 (6/304)	60 (3)	40 (3)	178
grp^1/grp^1	64 (5/167)	77 (3)	23 (3)	110
grp^1/Df	60 (7/111)	73 (5)	27 (5)	71

Syncytial embryos were fixed and stained for DNA, to determine the mitotic stage according to Edgar *et al.* [6]. Division cycle was determined by nuclear number and location (see Supplementary material). Mitotic indices are from cycles 1–11. Chromosome segregation data were from cycles 1–9 but the samples contained embryos mainly in cycles 6–9; before chromosome segregation includes prophase, prometaphase and metaphase; after segregation includes anaphase and telophase. As described previously, chromosomes do not condense properly onto the metaphase plate in *grp* embryos [3], even before cycle 9 (data not shown); consequently, it is harder to distinguish late prophase from metaphase in *grp* embryos, and hence our division of mitotic stages into before and after chromosome segregation. The data were from three and five different experiments, respectively, for *grp* and wild type.

As Grp promotes cyclin A degradation, we might detect genetic interactions between *grp* and cyclin A. We first tested whether *grp* mutants are sensitive to levels of cyclin A. Homozygous *grp* flies are viable but female sterile. When we introduced a single copy of a heat-inducible cyclin A transgene, however, we failed to recover homozygous grp^1 progeny (Table 2). Thus, even without induction, the presence of a heat-inducible cyclin A transgene (which by itself is viable as a heterozygote or homozygote) caused grp^1 to behave as a recessive lethal. The observed synthetic lethality suggests that the Grp deficiency sensitizes the fly to low levels of cyclin A expression from the transgene. In contrast to this strong interaction with increased cyclin A level, a reduction in the dose of cyclin A failed to suppress the grp^1 allele (data not shown and [9]). It is perhaps not surprising that reduction of cyclin A by half did not suppress a null allele of *grp*. Sibon *et al.* [9] found that reduction in the dose of cyclin A did suppress the lethality of *mei-41* mutations. The *mei-41* gene is a homolog of the gene *ATM*, which is mutated in the genetic disorder ataxia-telangiectasia; *mei-41* is thought to act upstream of *grp* in the checkpoint pathway, and mutations in *mei-41* result in a phenotype like *grp*, but less severe [9]. Importantly, the suppression of the *mei-41* embryonic lethality by cyclin reduction occurred without restoring interphase length. This result shows that the mitotic defect is not an inevitable consequence of premature entry into mitosis as previously thought [1]. We suggest that the mitotic defect is an anaphase failure as a result of defective metaphase destruction of cyclin A.

If Grp promotes the metaphase–anaphase transition, why is it dispensable at most stages of development? Before

Table 2

Synthetic lethality between *grp*^{1} mutation and a heat-inducible cyclin A transgene (hs–cyclin A).

Cross	Progeny	
	grp^1/*grp*^{1} (straight winged)	*grp*^{1}/CyO (curly winged)
grp^1/CyO X *grp*^{1}/CyO	50	112
	46	111
grp^1/CyO; hs–cycA/TM6 X *grp*^{1}/CyO; hs–cycA/TM6	0	69
	0	100

The number of *grp*^{1} homozygotes and heterozygotes recovered from crosses of *grp*^{1} flies with or without the hs–cyclin A transgene. The results of two independent crosses are shown for each experiment. The progeny from *grp*^{1}; hs–cyclin A/TM6 crosses included hs–cyclin A homozygotes and hs–cyclin A/TM6 heterozygotes. Data shown are from 25°C although similar results were obtained at room temperature or 18°C.

cell cycle 12, *grp* embryos exhibit a defective mitosis with delayed sister chromosome separation and, yet, mitosis is successful. From this we make two inferences: first, mitosis can tolerate a limited disruption in the timing of events and, second, as anaphase occurs in the absence of Grp, there must be a backup Grp-independent mechanism that promotes sister separation slightly later. The mitotic mis-coordination in *grp* embryos gets progressively more severe, and cyclin A levels progressively increase during the syncytial cycles [6]. This correlation, together with the ability of reduced cyclin A dose to suppress *mei-41* lethality, leads us to suggest that the consequence of a defect in the *grp*/*mei-41* pathway increases in severity as cyclin A increases, until anaphase fails at mitosis 12 and 13 (see, for example, Figure 2h–j).

The current model for Chk1 function involves the phosphorylation and inhibition of Cdc25, in part by binding of 14-3-3 protein to the phosphorylated Cdc25 and sequestration in the cytoplasm where it is ineffective in counteracting the nuclear kinases Wee1 and Mik1 [10,11]. Thus, inhibitory phosphorylation of Cdk1 prevents its activation and the cell arrests in G2. Although this action of Chk1 appears general, it is possible that Chk1 activity has other consequences. Indeed, there is no substantial accumulation of inhibitory phosphate on Cdk1 [6], and the Cdc25^{Stg} protein is constitutively present and nuclear during interphase of syncytial cycles 11–13 when a *grp*-dependent mechanism regulates the entry into mitosis ([6]; T.T.S., unpublished observations). The results presented here suggest that Grp may function to destabilize cyclin A. When a Grp-dependent cell-cycle checkpoint is induced by blocking S phase with aphidicolin in cleavage-stage *Drosophila* embryos, Cdc25^{Stg} is destabilized [9]. Thus, whether it is direct or indirect, Grp promotes the destruction of two cell-cycle proteins, Cdc25^{Stg} and cyclin A. We suggest that promotion of the metaphase–anaphase transition represents a second function of the *grp*/*mei-41* pathway, distinct from the checkpoint arrest of entry into mitosis. Nevertheless, a common mechanism might be involved because blocking entry into mitosis and promoting exit from mitosis both involve inhibition of cyclin/Cdk1 activity.

In summary, Grp is required for normal cyclin A turnover in the early *Drosophila* embryo. We have also found that *grp* mutant embryos show a delay in the timing of the metaphase–anaphase transition. Stable versions of cyclin A block chromosome separation at the metaphase plate, at least in cellularized *Drosophila* embryos, suggesting that proteolysis of cyclin A is required for this process [7,8]. Thus, the proposal that Grp activates cyclin A proteolysis can explain the mitotic phenotype as a consequence of at least temporary persistence of cyclin A.

Supplementary material

Additional methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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Supplementary material

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Supplementary materials and methods

Cyclin A turnover

Embryos were collected for 30 min and aged for 40 min to 1 h at room temperature to achieve cycles 4–8 (Figure 1a) or 8–12 (Figure 1b). In initial experiments, a fraction of embryos were fixed and stained for DNA (see below) and visualized to confirm that the expected cell-cycle number had been reached. In Figure 1a, embryos were dechorionated and permeabilized with octane using previously published procedures (for example, [S1]). Permeabilized embryos were incubated in Schneider's tissue culture medium containing 20 μg/ml cycloheximide for 30 min, with (+ col, + cyc) or without (+ cyc) a 20 min prior incubation in Schneider's medium containing 50 μg/ml colchicine. For controls, either untreated embryos or embryos that had been permeabilized and incubated in Schneider's media for 30 min were used, with no obvious difference. In both Figure 1a and 1b, approximately equal numbers of embryos were homogenized in HEMG buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM Na_2VO_4, 1 mM benzamidine, 0.2 mM PMSF, 2 μg/ml aprotinin, 1.5 mM DTT, 10% glycerol) and boiled in SDS gel loading buffer. Samples were separated on SDS gels and western blotted according to standard procedures. Western blots were probed with rabbit polyclonal antibodies against *Drosophila* cyclin A (1:700 dilution; [S2]) or monoclonal antibodies against *Drosophila* cyclin B (1:2 dilution; [S3]) or rabbit polyclonal antibodies against β-tubulin (Amersham). ECL (Amersham) detection was used for western blots. The blot was also stained with Ponseau (Sigma) to visualize proteins (see Figure 1b) before western blotting.

Antibody staining for PH3

Embryos were fixed for 20 min in PBS + 10% formaldehyde or 30 min in PBS + 3.7% formaldehyde, using standard procedures. DNA was stained with 10 μg/ml bizbenzamide (Hoechst 33258), and PH3 was detected with a purified rabbit polyclonal antibody (1:1000 dilution; Upstate Biotechnologies) against the epitope ARKS*TGGKAPRKQL (in the single-letter amino-acid code; the asterisk indicates that S is phosphorylated), which is present in three *Drosophila* histone H3 variants. In Figure 2 and Table 1, division cycle was determined from nuclei number (n = 2 for cycle 2; n = 4 for cycle 3, etc.) and nuclear location with respect to the embryo surface (in embryo interior through cycle 8, migration during cycle 9, surface reached at the end of cycle 9).

Fly stocks

The *grp* stock (grp^1/Cy) has been described before [S4,S5]. The *grp* mutation is a maternal-effect mutation; homozygous mutant mothers are identified by the lack of a CyO balancer and served as a source of *grp* embryos. The grp^1 allele was used in all experiments unless otherwise stated. Fly stock carrying the hs–cyclin A transgene on chromosome III has been described before [S6]. Flies carrying grp^1 and hs–cyclin A alleles were constructed using standard *Drosophila* techniques. The deficiency chromosome Df(2L)H20 carries breakpoints at 36A08-09;36E-01-02 (Bloomington Stock Center).

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